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p53 determines prognostic significance of the carbohydrate stem cell marker TF1 (CD176) in ovarian cancer

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Abstract

Introduction The oncofoetal Thomsen–Friedenreich (TF1, CD176) epitope is a carbohydrate cancer stem cell (CSC) antigen, and TF1-mediated cancer progression can be widely reversed by anti-TF1 antibodies. Particularly, CSC-like cells are regarded to be tumorigenic and chemoresistant. Aberrant p53 is probably the factor most closely associated with chemoresistance and tumour aggressiveness in ovarian tumours. We thus questioned whether TF1 in combination with p53 or as a single marker may be related to clinico-pathological features and survival of ovarian cancer patients.

Patients and Methods Both markers were quantified in ovarian cancer tissue ($n = 151$) by immunohistochemistry. p53 staining was subdivided into three subgroups [n (completely negative) $= 57$, n (moderately stained) $= 28$, n (overexpressing) $= 66$]. TF1 was scored as positive ($n = 30$) versus negative ($n = 121$).

Results Only in those cancers classified with moderate p53 staining—and thus most likely displaying with wild-type *TP53*—TF1 positivity turned out to be a predictor for shortened overall survival (univariate: $p < 0.001$, multivariate: $p = 0.001$). By screening 17 different protein markers for correlation with TF1, only mucin-1 emerged as a potential TF1 carrier protein.

Conclusion It is hypothesized that TF1 may confer tumour-promoting features, especially in a *TP53* wild-type genetic background. In addition, TF1 is an attractive immunotherapeutic target. Whether those cases classified as TF1 positive and at the same time as moderately stained for p53 might particularly benefit from a future anti-TF1 antibody treatment or from TF1 vaccination therapy remains to be determined.

Keywords Thomsen–Friedenreich · Epitope · CD176 · TF1 · p53 · Ovarian cancer · Prognosis

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Introduction

The oncofoetal Thomsen–Friedenreich (TF1, CD176) epitope is regarded to be a carbohydrate cancer stem cell antigen. Structure-wise TF1 resembles a Galβ1-3GalNAcα-residue linked to carrier proteins by O-glycosylation. Particularly, mucin-like glycoproteins have been demonstrated to carry the TF1 antigen (Dippold et al. 1990; Satyanarayana et al. 2001). In healthy adult tissue, the TF1 moiety is covered by a cryptic glyco-structure and was discovered to become exposed on the cell surface during the process of malignant transformation. Therefore, TF1 is hardly found in non-neoplastic adult tissue and thus may represent a tissue tumour marker of unique specificity (Karsten and

Goletz 2013). In line with this, established cancer stem cell markers in cancer initiating cells e.g. CD44 and CD133 were demonstrated to carry the TF1 epitope (Lin et al. 2011). The identification of TF1 on circulating tumour cells (CTCs) and the fact that TF1-mediated cancer cell adhesion and proliferation may be key during metastasis formation and cancer progression further support that TF1 expression might be a characteristic of CSCs (Karsten and Goletz 2013; Yu 2007; Schindlbeck et al. 2005). In addition, TF1-targeting antibodies have been shown to interfere with cell proliferation and migration in both cell culture and animal models (Almogren et al. 2012).

A common feature of CSCs is increased insensitivity towards anti-cancer drugs. With p53 being known to play a critical role in apoptosis and proliferation, loss of active p53 has become closely associated with multidrug resistance (Fraser et al. 2008). Since accumulation of the tumour suppressor protein p53 was observed in malignant cells (Dowell et al. 1994), it was hypothesized that certain mutations in *TP53* may either cause overexpression of non-functional p53 protein (Dowell et al. 1994; Soussi 2007) or may lead to total absence of the protein (Yemelyanova et al. 2011). Assessing p53 by immunohistochemistry instead of *TP53* mutation analysis has turned to be a well-established method (Zong et al. 2012; Figarella-Branger et al. 2011; Vereczkey et al. 2011; Ziolkowska-Seta et al. 2009) and has been intensively studied (Marks et al. 1991; Alsner et al. 2008). Yemelyanova et al. (2011) have established an immunohistochemical scoring system which has been proofed to identify almost 95 % of cases mutant for *TP53*.

Though TF1 has been dissected in tumours of the gastrointestinal tract, urinary bladder and breast for years, data on TF1 and interacting predictive markers in ovarian cancer are rather scarce. Since TF1 and p53 may be functionally related, it was aimed to determine whether there may be an association of TF1 and p53 in ovarian cancer. Further, to identify potential TF1 carrier proteins, a correlation analysis of TF1 and 17 different protein markers has been performed. Finally, it was analysed whether TF1 in combination with p53 or as a single marker may be related to clinico-pathological features and overall survival in ovarian cancer patients.

Materials and methods

Patients and specimen characteristics

Ovarian cancer specimens stemming from 151 patients were randomly selected from the archives of the Department of Obstetrics and Gynaecology, Ludwig Maximilians University of Munich, Germany. Patients had undergone surgery between 1990 and 2002, and no patient had received neo-adjuvant chemotherapy. Patients were staged according to the FIGO classification [n (FIGO I and II) = 45 (30.0 %), n (FIGO $\geq$ III) = 105 (70.0 %)], and almost one half of the cases was diagnosed with high-grade carcinoma (G 1 and 2: n = 86, 61.9 %; $G \geq 3$: n = 53, 38.1 %). Data on lymph node involvement were available in 92 cases [lymph node-negative patients: n = 42 (45.7 %); lymph node-positive patients: n = 50 (54.3 %)]. Median patient age was 58.6 years (range 20.7–88.1 years), and histological subtypes were distributed as follows: n (serous) = 106 (70.2 %), n (endometrioid) = 20 (13.2 %), n (clear cell) = 12 (7.9 %), n (mucinous) = 13 (8.6 %).

Study design

Patients were recruited at the Department of Gynaecology and Obstetrics of the Ludwig Maximilians University of Munich, Germany, between 1990 and 2002. Women only diagnosed for benign or for borderline tumours of the ovary were excluded from the study. Clinical as well as follow-up data were retrieved retrospectively from patients' charts and from the Munich Cancer Registry. Overall mean survival of the cohort was 7.1 years (95 % CI 6.0–8.4 years), and mean follow-up time was 11.0 years (95 % CI 9.8–12.1 years). The outcome assessed was patient overall survival.

Ethical approval

All patient data were fully anonymised, and the study was performed according to the standards set in the Declaration of Helsinki 1975. All tumour tissue used was leftover material that had initially been collected for histopathological diagnostics. All diagnostic procedures had already been fully completed when samples were retrieved for the study. The current study was approved by the Ethics Committee of the Ludwig Maximilians University, Munich, Germany (approval number 227-09). Authors were blinded for clinical information during experimental analysis.

Assay methods

Formalin-fixed, paraffin-embedded tissue sections were stored at room temperature. Slides were dewaxed in xylol and rehydrated in a descending series of alcohol. Epitope retrieval (for p53 staining only) was performed in citrate buffer (pH 6) in a pressure cooker for 5 min. Sections were rinsed in distilled water and equilibrated in phosphate-buffered saline. Antibodies (p53: mouse IgG2aK, Oncogene, Rockville, MD; TF1: Nemod-TF1 (mouse IgM, kappa), Glycotope, Berlin, Germany) were incubated for 90 min at room temperature (p53, diluted 1:200) or overnight at 4 °C (TF1, diluted 1:100). Primary antibodies were detected by using the Vectastain Elite mouse kit (Vector Labs, Burlingham, CA) as per manufacturer's protocol. In case of TF1

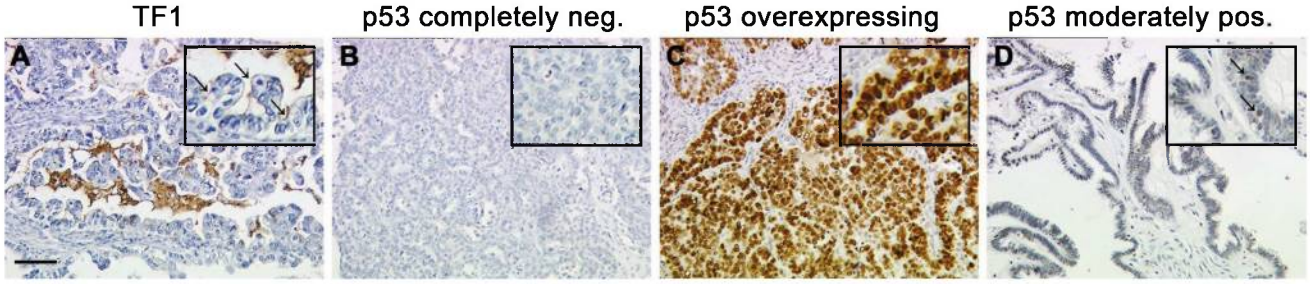

Fig. 1 Detection of TF1 and p53 by immunohistochemistry. Representative photomicrographs of TF1 (**a**) and p53 (**b** completely negative staining, **c** overexpression, **d** moderately positive staining) are shown. *Arrows* in **a** indicate TF1 immunoreactivity on the apical cell surface, while *arrows* in **d** mark positive nuclear p53 signals. *Scale bar* in **a** equals 100 μm and applies to **a**–**d**. *Inserts* are magnified by twofold

staining, an IgM secondary antibody (Linaris, Dossenheim, Germany) was used. Finally, slides were dehydrated in an ascending series of alcohol and covered with Eukitt (Sigma-Aldrich, St. Louis, MO).

Appropriate positive (placenta and breast cancer tissue) and negative controls were included in each experiment. Negative controls were treated with isotype-matched IgG (Dako, Hamburg, Germany) instead of the primary antibody. Immunoreactivity was quantified by applying a well-established semiquantitative scoring system (IR-score; Remmele's score) by two independent observers by consensus. This scoring method has already been used in numerous studies (Ditsch et al. 2013; Ditsch et al. 2012a, b; Lenhard et al. 2011) and quantifies immunoreactivity by multiplication of optical staining intensity (graded as 0: no, 1: weak, 2: moderate and 3: strong staining) and the percentage of positively stained cells (0: no staining, 1: ≤10 % of the cells, 2: 11–50 % of the cells, 3: 51–80 % of the cells; and 4: ≥81 % of the cells). Since TF1 is only produced by certain tumour cells, slides assigned an IRS higher than IRS = 0 were scored as positive. The cut-offs for determining p53 negative versus moderate versus overexpression were based on those set by Yemelyanova et al. (2011) and adapted to the IR scoring system (completely negative: IRS 0, overexpression: intensity >1 and percentage of stained cells >50 %, moderate staining = remaining IR scores).

Antibody stainings (FSHR, LHR, hCG, ERα, ERβ, PRA, PRB, GPER, Her2, GdC15, GdQ13, PankoMabGEX^{mem}, PankoMabGEX^{cyt}, 115D8, HMFG1, VU3C6, VU4H5) that were correlated with TF1 (Table 2) were already published by our group (Lenhard et al. 2011; Dian et al. 2013; Engelstaedter et al. 2012; Heublein et al. 2013a, b; Lenhard et al. 2012a, b).

Statistical analysis methods

This study has been carried out according to the REMARK (Reporting Recommendations for Tumour Marker Prognostic Studies) criteria (McShane et al. 2005). The IBM statistical package SPSS (version 22) was used to test data for statistical significance. Chi-square testing and Spearman's correlation analysis were performed. Survival times were compared by using Kaplan–Meier graphics, and differences in patient overall survival were tested for significance by using Chi-square statistics of the log-rank (Mantel–Cox) test. Data were assumed to be statistically different in case of $p < 0.05$.

Results

Study cohort

The TF1 epitope was only detected in 30 (19.9 %) out of the 151 specimens examined. TF1 expression was restricted to the apical cell surface, and TF1 was also found shed into intercellular spaces (Fig. 1a). While serous carcinomas overexpressed TF1 as compared to non-serous (i.e. endometrioid, mucinous, clear cell) ones [TF1 positive fraction (serous) = 26/106, TF1 positive fraction (non-serous) = 4/45; $p = 0.028$], no relation of TF1 positivity and tumour grade, stage, lymph node status or patient age was observed (Table 1). Immunostaining of p53 was assessed by a scoring system which has been delineated from a scoring method published previously (Yemelyanova et al. 2011). While 37.7 % ($n = 57$) of cases were scored as completely negative (Fig. 1b), almost half of all samples ($n = 66$, 43.7 %) overexpressed p53 (Fig. 1c). Moderate p53 staining (Fig. 1d) was found in 28 cases (18.5 %). p53 was differentially expressed among histological subtypes ($p = 0.047$), FIGO stage ($p = 0.048$) and patient age ($p = 0.014$), while no difference was found regarding tumour grade or lymph node status. Further, there was no correlation between TF1 and p53 expression.

Table 1 Distribution of clinico-pathological variables

	TF1		*p*	p53			*p*
	Neg.	Pos.		Completely negative	Moderately positive	Overexpression	
Histology							
Serous	80	26	0.028	39	15	52	0.047
Other	41	4		18	13	14	
FIGO							
I/II	34	11	0.373	22	10	13	0.048
III/IV	86	19		34	18	53	
Grade							
G1/2	67	19	0.192	35	15	36	0.229
G3	46	7		14	12	27	
pN							
N0	31	11	0.229	21	9	12	0.084
N1	42	8		15	10	25	
Age							
≤60 years	65	15	0.682	35	19	26	0.014
>60 years	55	15		22	9	39	

Potential TF1 carrier proteins in ovarian cancer

The TF1 epitope was found to be exposed on mucin-like glycoproteins (Dippold et al. 1990; Satyanarayana et al. 2001). Within the current analysis, Spearman's correlation was performed thus to determine potential TF1-associated (carrier) proteins out of a panel of 17 candidate molecules. All correlation analyses were performed in p53 moderately positive, p53 completely negative and in p53 overexpressing cases. Though gonadotropin receptors and hCG are known to be highly glycosylated, no correlation of TF1 and these markers was observed. Further (growth) hormone receptor expression did not correlate with TF1. Different glycodelins were not associated with TF1. However, in p53 negative ($p = 0.016$), p53 moderately positive ($p = 0.003$) and p53 overexpressing ($p = 0.005$) cases, a strong positive correlation of TF1 and mucin-1 (MUC1) as detected by the established MUC1 antibody VU-3C6 was observed (Table 2).

TF1 is an independent prognosticator in cancers classified as moderately stained for p53

Regarding the whole study panel TF1 was not associated with overall survival. Patients were then grouped according to their p53 immunoreactivity and were classified as completely negative, moderately stained for p53 or overexpressing. Survival times of these three subgroups were significantly different ($p = 0.016$, Fig. 2a). Overall survival of TF1 negative versus positive cases was compared by Kaplan–Meier analysis in the subgroups specified above. However, only in case of moderate p53 staining TF1 was of prognostic significance and predicted shortened median overall survival [95 % CI (TF1 negative): 5.47–12.1 years; 95 % CI (TF1 positive): 0.17–3.01 years; $p < 0.001$, Fig. 2d].

TF1 expression in cases classified with moderate p53 staining was further studied. Multivariate Cox-regression analysis (Table 3) revealed TF1 positivity to serve as a negative prognosticator ($p = 0.001$) for overall survival independent of tumour histology, stage, grade or patient age. Among TF1 grading ($p = 0.032$) emerged to be an independent prognosticator in those cancers categorized as moderately expressing p53.

Discussion

This study detected TF1 to be expressed in a small fraction of ovarian cancer cases and to independently predict shortened overall survival in patients diagnosed for ovarian cancer classified as moderately stained for p53.

Though p53 was overexpressed in about half of all samples, TF1 positivity was only present in a small fraction of cases and only few cells per tumour sample appeared to stain positive for TF1. Hypothesizing that TF1 may be a stem cell antigen or may be linked to stem cell markers (e.g. CD44) by O-glycosylation, the expression pattern described above was expected (Lin et al. 2011). Since the nature of the current study was rather descriptive, it is not known whether these TF1 positive cells may indeed be capable of initiating neoplastic lesion in nude mice—which, if proven true, would confirm them to be CSCs. Interestingly, a former study of our group revealed TF1 to

Table 2 Correlation analysis

	Gonadotropin receptor			Hormone receptors						Glycodelin			Mucin-1/CD227					
	LHR	FSHR	HCG	ER alpha	ER beta	GPER	PRA	PRB	Her2	GlycC15	GlycQ13	Glyc2	Panko^{Mem}	Panko^{Cyt}	115D8	HMFG1	VU3C6	VU4H8
p53 negative																		
TF1																		
cc	−.017	−.107	.209	.221	−.023	.164	.048	.166	−.161	.014	.042	−.209	.008	−.007	.009	−.045	.322	.003
p	.899	.438	.126	.099	.867	.223	.725	.218	.237	.921	.760	.122	.958	.958	.951	.742	.016	.985
n	57	55	55	57	56	57	57	57	56	56	56	56	52	52	55	57	56	54
p53 moderately positive																		
TF1																		
cc	.057	−.003	.108	−.122	.374	−.326	−.102	−.135	nd	−.149	−.204	.002	.056	.169	.217	.249	.547	.297
p	.774	.988	.608	.536	.055	.097	.605	.494	nd	.450	.297	.993	.780	.398	.297	.211	.003	.140
n	28	28	25	28	27	27	28	28	27	28	28	28	27	27	25	27	27	26
p53 overexpression																		
TF1																		
cc	.024	.217	−.069	.043	.072	.115	.026	−.135	.039	−.014	−.017	.145	.080	.206	.088	−.042	.345	.015
p	.850	.087	.596	.730	.567	.359	.840	.278	.760	.912	.893	.249	.542	.114	.483	.738	.005	.908
n	64	63	61	66	66	66	65	66	65	65	65	65	60	60	65	65	65	66

Immunoreactivity of 17 different potential TF1 carrier proteins was correlated with TF1 positivity using Spearman's rho in three subgroups (completely negative, moderate staining and overexpression). cc = correlation coefficient, p = two-tailed significance, LHR = luteinizing hormone receptor, FSHR = follicle-stimulating hormone receptor, hCG = human chorionic gonadotropin, ERα/β = oestrogen receptor alpha/beta, PRA/B = progesterone receptor A/B, Her2 = epidermal growth factor receptor 2, GPER = G-protein coupled oestrogen receptor, GdC15/Q13 = glycodelin as detected by anti-Gd-peptide antibodies, GdA = glycodelin as detected by a carbohydrate-specific antibody, PankoMabGEX$^{mem/cyt}$ = PankoMab (TA-MUC1) membrane/cytoplasmic staining, 115D8/HMFG1/VU-3C6/VU-4H5 = MUC1 antibodies detecting different epitopes on MUC1

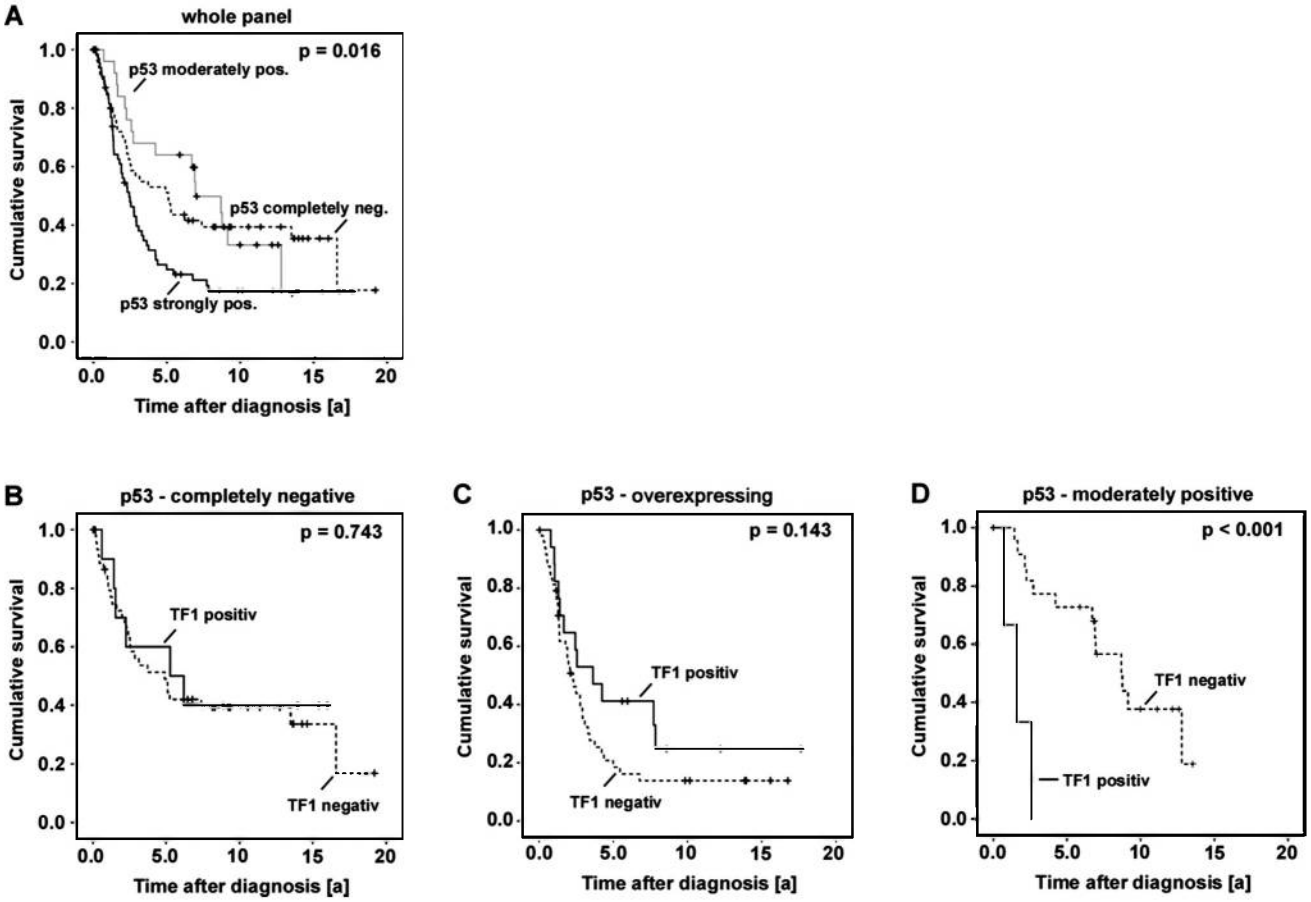

Fig. 2 TF1 predicts shortened survival in ovarian cancers classified as moderately stained for p53. Survival times were plotted as Kaplan–Meier graphs. Overall survival of subgroups as determined by p53 immunoreactivity was compared (**a**). The study sample was then subdivided into groups characterized as p53 completely negative (**b**), p53 overexpressing (**c**), p53 moderately stained (**d**) and survival of TF1 negative versus positive patients was contrasted

Table 3 Multivariate survival analysis in ovarian cancer cases moderately stained for p53

Covariate	Coefficient (b_i)	[HR Exp(b_i)]	95 % CI		p value
			Lower	Upper	
Histology (ser. vs. other)	1.198	3.312	.832	13.180	.089
FIGO (II vs. III, IV)	1.840	6.294	1.171	33.835	.032
Grade (G1, G2 vs. G3)	.894	2.446	.665	8.999	.178
Patient age (≤60 vs. >60 years)	.422	1.525	.431	5.400	.513
TF1 (negative vs. positive)	3.164	23.66	3.853	145.3	.001

be present in a similar low fraction of cancer cells in breast cancer tissue section, but more important found that almost all disseminated tumour cells do express TF1 (Schindlbeck et al. 2005, 2007). As those disseminated tumour cells are suspected to be important for distant metastatic spread and as metastasis formation is a characteristic feature of CSC, the hypothesis of TF1 to represent a new CSC marker is further supported.

So far several glycoproteins have been identified to function as TF1 carrier proteins with most of them being classified as mucin-like glycoproteins. However, in the case of ovarian cancer (to the best of our knowledge) no published data on potential TF1 carrier proteins exist. Thus, to screen for candidate proteins we performed Spearman's correlation analysis using 19 protein markers including TF1. Since MUC1 as detected by VU-3C6 was discovered to correlate with TF1, we hypothesize that MUC1 may be a candidate to expose the TF1 epitope on ovarian cancer cells. This is supported by the fact that MUC1 also serves as a TF1 carrier molecule in gastric and breast carcinoma

(Masuzawa et al. 1992; Lloyd et al. 1996). With MUC1 itself having been implicated in cancer cell adhesion and progression in ovarian cancer (Dian et al. 2013; Quin and McGuckin 2000), effects of anti-TF1 antibodies on MUC1 action and vice versa remain to be investigated.

Whether determination of p53 by immunohistochemistry may be sufficient to conclude on *TP53* mutation is controversial. Though immunohistochemistry may not detect all the *TP53* mutations, it is widely used, easy to perform and cost-effective (Zong et al. 2012; Figarella-Branger et al. 2011; Vereczkey et al. 2011; Ziolkowska-Seta et al. 2009). In addition, our data on p53 overexpression rate are similar to those published by others (Choudhury et al. 2012; de Graeff et al. 2009), and more importantly, the results presented here are based on p53 expression regardless whether this staining pattern per se is due to *TP53* mutation or not.

The presence of the TF1 epitope has been associated with patients' prognosis in different types of cancer so far. Though most studies reveal that expression of TF1 predicts shortened disease free or overall survival (Baldus et al. 2000, 2001; Takanami 1999), others found TF1 to be associated with favourable prognosis (Schindlbeck et al. 2007; Choufani et al. 1999). Though appearing contradictive, both observations may be interpreted by TF1-mediated biological action. Since anti-TF1 antibodies effectively blocked cell proliferation, tumour cell adhesion and migration, TF1 itself may comprise pro-tumorigenic actions and may thus be associated with worse prognosis (Rittenhouse-Olson 2007; Ferguson et al. 2014). On the other hand, TF1 is assumed to induce anti-tumour immune response thus may enable the host to exert a more effective anti-tumour immune defence (Schindlbeck et al. 2007). This is an interesting observation in terms of developing anti-cancer vaccines for personalized therapy and apart from that may help to interpret how TF1 may be related to favourable prognosis in some few studies. The only study dealing with the prognostic significance of TF1 in ovarian cancer was performed in a sample of 30 patients and remarkably demonstrated TF1 to be associated with worse prognosis (Ghazizadeh et al. 1990). By confirming these results in ovarian cancer patients moderately stained for p53, we added further evidence that regarding ovarian cancer TF1 may represent an interesting predictive and potentially prognostic tissue marker. However, the significance of our study is limited by the quite small sample size, and this particularly becomes apparent in multivariate analysis. Whether those cases classified as TF1 positive and at the same time as moderately stained for p53 might particularly benefit from a future anti-TF1 antibody treatment or from a TF1 vaccination therapy remains to be determined.

Conclusions

The current study highlighted the oncofoetal carbohydrate TF1 (CD176) antigen to be an independent prognostic marker for overall survival of those ovarian cancer patients classified as moderately stained for p53. It is hypothesized that TF1 may confer to tumour-promoting features, especially in a p53 wild-type genetic background. Whether this observation may impact on efficacy of future anti-TF1 antibody treatment strategies or TF1 vaccination protocols needs to be determined.

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Compliance with ethical standards

Conflict of interest The authors declare that no competing interests exist.

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